FEBS 29027 FEBS Letters 578 (2004) 357–362

Specific interaction between S6K1 and CoA synthase: a potential link between the mTOR/S6K pathway, CoA biosynthesis and energy metabolism

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Received 26 August 2004; revised 12 October 2004; accepted 28 October 2004

Available online 16 November 2004

Edited by Gianni Cesareni

Abstract **Ribosomal protein S6 kinase (S6K) is a key regulator of cell size and growth. It is regulated via phosphoinositide 3-kinases (PI3K) and the mammalian target of rapamycin (mTOR) signaling pathways. We demonstrate for the first time that CoA synthase associates specifically with S6K1. The association was observed between native and transiently overexpressed proteins in vivo, as well as by BIAcore analysis in vitro. The sites of interaction were mapped to the C-terminal regions of both CoA synthase and S6K1. In vitro studies indicated that the interaction does not affect their enzymatic activities and that CoA synthase is not a substrate for S6 kinase. This study uncovers a potential link between mTor/S6K signaling pathway and energy metabolism through CoA and its thioester derivatives, but its physiological relevance should be further elucidated.**

Keywords: Ribosomal protein S6 kinase; S6k1; CoA synthase; mTOR

1. Introduction

Ribosomal protein S6 kinase (S6K) is involved in signaling pathways, which regulate cell growth, size and energy metabolism. Two isoforms of S6K have been identified (S6K1 and S6K2) [1–3]. Both kinases are activated in response to mitogenic stimuli and nutrients via PI3-K and mTOR signaling pathways. It is believed that conformational changes induced by multiple S/T phosphorylations open the structure, making both N- and C-terminal regulatory domains available for protein–protein interactions. So far, only a small number of S6K-binding partners have been identified, including PKC, PDK1, protein phosphatase PP2A, cytoskeletal protein neurabin and small GTP-binding proteins Rac and Cdc42 [4–8].

Ribosomal protein S6 is the best studied physiological substrate of S6Ks in vivo. In addition, the transcriptional activator CREM, elongation factor 2 kinase and the regulator of apoptosis Bad 1 were also found to be phosphorylated by S6K1, but the physiological relevance of these modifications remains to be further investigated [9–11].

Knockout studies in mice and flies provided the evidence for the involvement of S6K in the regulation of cell growth, size and glucose homeostasis [12]. Moreover, the mTor/S6K pathway has been recently implicated in energy metabolism. The link became apparent through the study of tumor suppressor proteins, TSC1 (hamartin) and TSC2 (tuberin), which are mutated in the relatively common genetic disorder, tuberous sclerosis [13,14]. A recent paper by Inoki et al. [14] indicated that TSC2 plays a key role in a pathway, which maintains cellular energy homeostasis. Another potential link to the regulation of energy metabolism via mTor/S6K pathway became evident with the molecular cloning of CoA synthase, which was identified as an S6K-binding partner in a yeast two-hybrid screen [15]. Biochemical and mutational analyses of CoA synthase indicated that it possesses 4′-phosphopantetheine adenylyltransferase and dephospho-CoA kinase activities, which mediate to the last two steps in CoA biosynthesis. Transient expression studies and confocal microscopy allowed us to demonstrate that CoA synthase is associated with mitochondria [16]. In this study, we demonstrate for the first time specific interaction between CoA synthase and S6K1 by co-immunoprecipitation studies in mammalian cells and by BIAcore analysis in vitro. The C-terminal regions of CoA synthase and S6Ks mediate the interaction between both proteins. CoA synthase is not a substrate for S6K in vitro and its activity is not affected by rapamycin or LY294002 in vivo. The physiological relevance of the identified interaction is currently under investigation.

2. Materials and methods

2.1. Cell cultures and antibodies

HEK293 and MCF7 cell lines were purchased from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics (Invitrogen). Anti-Myc (9E-10) monoclonal antibody was purchased from Santa Cruz. Anti-phospho S6 (pSer 240/244) and anti-phospho S6K (pThr 389) antibodies were from Cell Signaling. Monoclonal antibody to the EE-tag was a gift from Julian Downward. The production of monoclonal and polyclonal antibodies directed against S6K1 and CoAsy was described previously [15,17,18].

2.2. Plasmid construction and expression studies

The full-length coding sequence of CoAsy, ΔNCoASy and ΔC-CoASy deletion mutants was amplified by PCR and cloned into pcDNA3.1 vector (Invitrogen) in frame with the C-terminal Myc-tag

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0014-5793/$22.00
doi:10.1016/j.febslet.2004.10.091

epitope. The pcDNA3.1 plasmids containing S6K1, S6K2, ΔN-S6K1 and ΔC-S6K1 in frame with the N-terminal EE-tag epitope were described previously [4]. Transient transfection of HEK293 cells was performed using Polyfect (Qiagen).

2.3. Immunoprecipitation and immunoblot analysis

HEK 293 cells were starved in serum-free DMEM for 24 h and then stimulated with 10% FBS or 1μM PMA for 1 h. Cells were washed with ice cold phosphate-buffered saline and extracted with lysis buffer, containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 1% (v/v) Nonidet P-40, 2 mM EDTA, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate and a mixture of protease inhibitors (Roche). Cell lysates were centrifuged at $10000 \times g$ for 30 min at 4 °C, and recombinant Myc-CoAsy or EE-S6K1/2 was immunoprecipitated with the anti-Myc or anti-EE monoclonal antibodies immobilized on protein A–Sepharose beads (Amersham). Immune complexes were washed three times with lysis buffer and used for in vitro kinase assay or immunoblot analysis. Immunoblot analysis was performed using PVDF membrane and enhanced chemiluminescence system, as described previously [4].

2.4. Analysis of S6 kinase and CoA synthase activities in vitro

Exponentially growing MCF-7 cells were starved in serum-free and phenol red-free DMEM for 24 h and then stimulated with 10% FBS for 1 h. Rapamycin (150 nM) and LY294002 (60 μM) were added to starved cells, 30 min before stimulation. Cell lysates were prepared as described above and the extracts immunoprecipitated with the C-terminal anti-CoAsy antibodies. The immunoprecipitates were analyzed for S6K and CoA synthase activities, as described previously [4,15]. Expression of EE-S6K1 and CoAsy-Myc in insect cells and affinity purification of recombinant proteins are presented in [4,16].

2.5. Characterization of S6K1 and CoASy elution profiles by gel filtration

Gel filtration chromatography was performed on a Superose 6 10/300 column (Amersham) connected to an AKTA FPLC system. The equilibration, lysis and elution buffer contained 25 mM Tris, pH 8.5, 200 mM NaCl and a cocktail of protease inhibitors (Roche). Five hundred microgram of rat brain soluble fractions after lysis (10 min on ice) and centrifugation ($15000 \times g$ for 30 min at 4 °C) was applied on a column equilibrated with lysis buffer. After chromatography, eluted fractions were subjected to SDS–PAGE and immunoblot analysis with anti-S6K and anti-CoAsy antibodies. Apparent molecular weight (mass) of eluted proteins was calculated using gel filtration standard proteins, including thyroglobulin (670 kDa), bovine gammaglobulin (158 kDa), chicken ovalbumin (44 kDa), equine myoglobin (17 kDa), and vitamin B_{12} (1.35 kDa). Void volume corresponded to the elution of Blue Dextran 2000.

2.6. Biosensor experiments

The basic operating procedures of the surface plasmon resonance BIAcore biosensor (BIACORE AB, Uppsala) have been published [19]. A biosensor chip (CM-5, BIACORE AB) was used to immobilize CoASy, using the amine coupling kit (BIACORE AB). CoAsy was applied at a concentration of 7 μg/ml in 20 mM acetate buffer, pH 4.5, until approximately 12000 Resonance Units (RU) were obtained. A separate surface was similarly coated with the dephospho CoAsy kinase, which served as a control. All interactions were carried out in 20 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% Tween 20, and 4 mM dithiothreitol. Varying concentrations of S6K in the above buffer (5–500 nM) were injected over the two surfaces at a flow rate of 10 μl/min at 25 °C, and the response at equilibrium was recorded. The response from the control surface was subtracted and the data plotted as Resonance Units (RU) vs S6K concentration. The equilibrium dissociation constant K_D was calculated by fitting the data to the equation $R = R_{max} \times C/(K_D + C)$, where R is the response at equilibrium, R_{max} the maximum response level and C the concentration of protein.

3. Results

The yeast two-hybrid screen was used to isolate binding partners towards wild type and activated forms of S6K1. This approach allowed us to identify several novel S6K1 interacting molecules. One of them was found to encode a novel protein. It was identified in two independent yeast two-hybrid screens with mouse embryo and HeLa cDNA libraries. In our initial studies, we focused on molecular cloning, biochemical and functional characterization of this protein. We found that this protein mediates the last two steps in CoA biosynthesis via 4′-phosphopantetheine adenylyltransferase and dephospho-CoA kinase activities and termed it CoA synthase [15]. Furthermore, we demonstrated that CoA synthase is localized on the outer mitochondrial membrane and that its activity is strongly activated by phospholipids [16]. Molecular cloning and characterization of CoA synthase provided us with necessary reagents required to study the specificity of interaction with S6K1 and its functional consequence in mammalian cells.

3.1. CoAsy forms a complex with S6K1/S6K2 in mammalian cells

To examine whether CoA synthase can interact directly with S6K1 or S6K2 in mammalian cells, we tested the association between transiently overexpressed and endogenous proteins. Initially, full-length CoAsy-Myc and EE-tagged S6K1 or S6K2 were transiently coexpressed in HEK293 cells. After starvation for 24 h, cells were stimulated with FBS or phorbol 12-myristate 13-acetate (PMA) for 1 h. Cell extracts were subjected to immunoprecipitation with anti-EE antibody, the immune complexes separated by SDS–PAGE and analyzed by Western blotting with anti-Myc antibody. Fig. 1A clearly indicates that CoASy-Myc protein co-immunoprecipitates with anti-EE antibody, indicating specific interaction between CoAsy and S6K1 or 2. It is important to note that the interaction is inducible upon stimulation with serum and PMA, but still occurs in serum-starved cells. The immunoprecipitated band matches a ~60 kDa protein, which corresponds to CoASy-Myc in total lysates of cells transfected with CoASy expression plasmids. No such protein band was detected in control immunoprecipitations (Fig. 1A, lanes 1–3). Furthermore, the interaction of endogenous proteins was investigated. For this purpose, we used MCF7 cells, since this breast cancer cell line overexpresses both S6K1 and CoAsy at relatively high levels [15,17]. In this study, we tested the presence of S6K activity in anti-CoAsy immunoprecipitates. Fig. 1B shows that S6K activity towards ribosomal protein S6 is specifically immunoprecipitated with anti-CoASy antibodies. No kinase activity was found to be associated with protein A–Sepharose beads alone. Moreover, immune complexes contain kinase activity which is induced in response to serum and is completely inhibited by indirect inhibitors of S6K, rapamycin (mTor inhibitor) and LY294002 (PI3-K inhibitor). The sensitivity to rapamycin and LY 294002 indicates that immunoprecipitated activity belongs to S6K but not other kinases, which can phosphorylate S6 protein in vitro including RSK.

3.2. Mapping the interaction domains in CoAsy and S6K1

To map the S6K1 interaction domain in CoAsy, two deletion mutants were constructed in pcDNA3.1 plasmid. One mutant (ΔC CoAsy-Myc) lacks the C-terminally localized dephospho-CoA kinase domain. In the second mutant (ΔN CoAsy-Myc), the N-terminal regulatory domain which mediates the interaction with mitochondria was deleted (Fig. 2A). Both deletion mutants were transiently expressed in Hek293 cells together with the EE-tagged version of the wild type

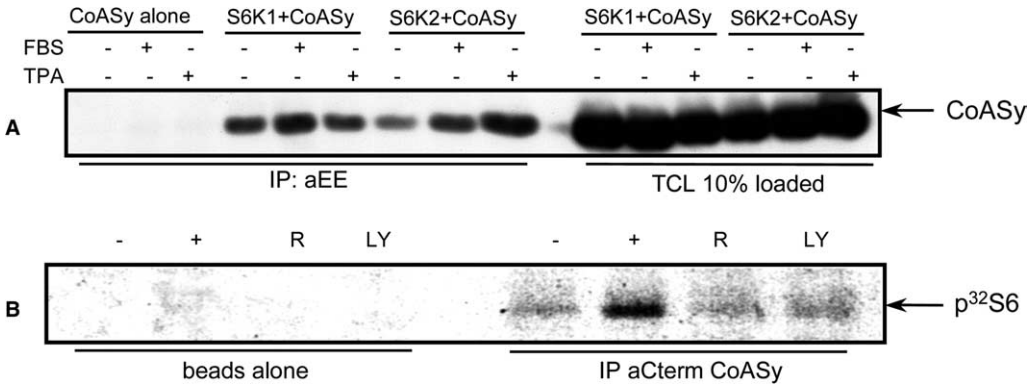

Fig. 1. CoA synthase interacts with S6K1 and 2 in serum starved and stimulated cells. (A) Specific interaction between transiently overexpressed CoAsy and S6K1 and 2. HEK293 cells were transfected with pCDNA3.1/CoAsy-Myc alone, pCDNA3.1/CoAsy-Myc and pcDNA3.1/EE-S6K1 or pCDNA3.1/CoAsy-Myc and pcDNA3.1/EE-S6K2. After transfection, cells were serum-starved for 24 h and stimulated for 60 min with 10% FCS or 1 μM PMA. The supernatants of lysed cells were immunprecipitated with anti-EE antibody and immune complexes or total cell lysates analyzed by Western blotting with anti-Myc antibody. (B) S6K activity co-immunoprecipitates with CoAsy in MCF7 cells. Serum starved MCF-7 cells were stimulated with 10% FBS for 1 h. Rapamycin (150 nM) and LY294002 (60 μM) were added to starved cells 30 min before stimulation. Cell lysates were divided and subjected to immunoprecipitation with the C-terminal anti-CoAsy antibodies or protein-A beads alone. The immune complexes and control beads were used for in vitro S6K kinase assay, using ribosomal protein S6 as a substrate. The reaction products were separated by SDS–PAGE and analyzed by autoradiography.

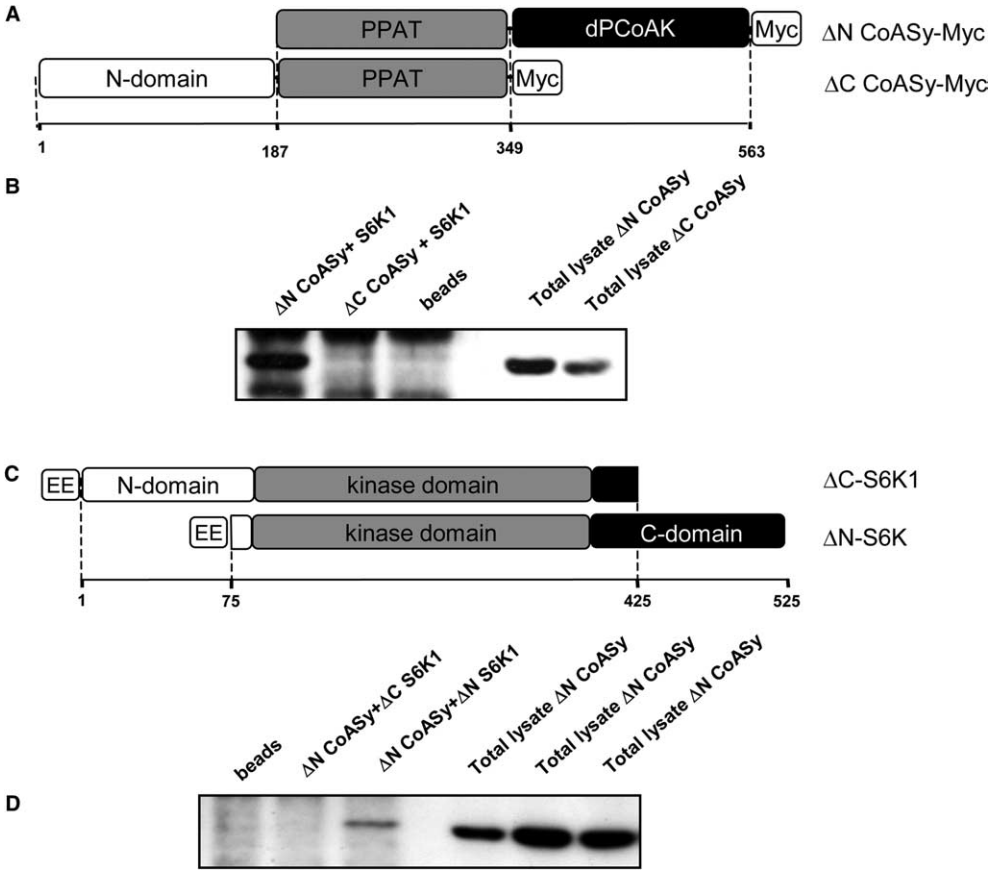

Fig. 2. The interaction between CoAsy and S6K1 is mediated by their C-term regulatory domains. (A) Modular representation of CoAsy deletion constructs, which were used in this study. The length of the fragments is marked in aa below. The Myc-tag epitope is located at the C-terminus. (B) The C-terminal region of CoAsy associates with S6K1. HEK293 cells were transfected with pcDNA3.1/EE-S6K1 and pCDNA 3.1/ΔN CoASy-Myc or pCDNA 3.1/ΔC CoASy-Myc plasmids. The anti-EE antibody was used for immunoprecipitation assay and the anti-Myc antibody for Western blotting. (C) Modular representation of the N- and C-terminal deletion mutants of S6K1 used in this study. The length of the fragments is marked in aa below. The EE-tag epitope is located at the N-terminus. (D) The C-terminal autoinhibitory domain of S6K1 interacts with CoAsy. HEK293 cells were transfected with pCDNA3.1/ΔNCoASy-Myc and pcDNA3.1/EE-ΔC S6K1 or pcDNA3.1/EE-ΔN S6K1 plasmids. The anti-Myc antibody was used for immunoprecipitation and the anti-EE antibody for Western blotting.

S6K1. Cell extracts were immunoprecipitated with anti-EE antibody and the immune complexes analyzed by immunoblotting with anti-Myc antibody. Fig. 2B shows that both deletion mutants were well expressed in Hek293, but only ΔN CoAsy-Myc readily co-precipitated with S6K1. No non-specific binding of exogenously expressed proteins to protein A–Sepharose beads coupled with control IgG was observed. These results indicate that the C-terminal region of CoAsy which possesses dephospho-CoA kinase domain mediates the interaction with S6K1. To determine the region in S6K1, which mediates the binding with CoA synthase, we used ΔN and ΔC deletion mutants of S6K1, schematically presented in Fig. 2C. In this experiment, both S6K1 mutants were transiently expressed together with the ΔN CoAsy-Myc, as it binds more efficiently to S6K1 than the full-length CoAsy. Transiently expressed ΔN CoAsy-Myc was immunoprecipitated with anti-Myc antibody followed by Western blot analysis with anti-EE antibody. As shown in Fig. 2D, the interaction is observed between ΔN CoAsy-Myc and ΔN S6K-EE, but not with ΔC S6K-EE, indicating the necessity of the C-terminus of S6K1 for the interaction with CoASy. We also detected the binding of the full-length CoAsy-Myc to ΔN S6K-EE, but it was less efficient (data not shown). Taken together, these data indicate that the interaction between CoA synthase and S6K1 is mediated by their C-terminal regions, containing dephospho-CoA kinase domain and the regulatory autoinhibitory region, respectively.

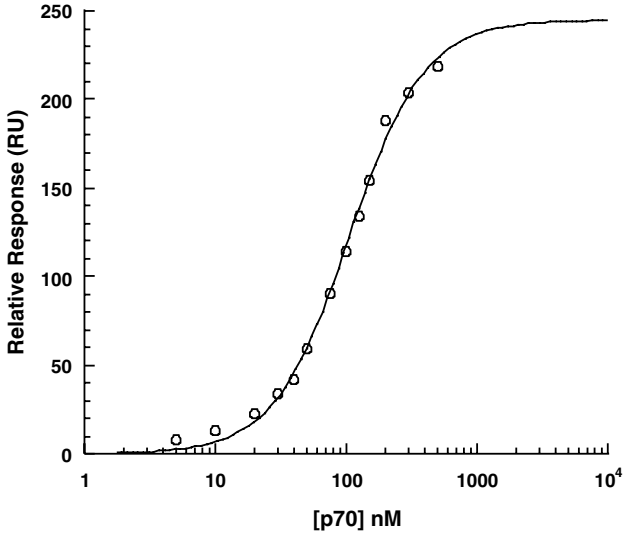

Fig. 3. BIAcore analysis of CoAsy and S6K 1 interaction. Specific binding of S6K1 to immobilized CoAsy measured by a surface plasmon resonance biosensor (BIACORE). The estimated equilibrium dissociation constant (K_D) of the interaction between CoASy and S6K is 184 nM.

3.3. Real-time analysis of interaction between the S6K and CoASy

A surface plasmon resonance biosensor (BIACORE) was used to monitor the specificity and the affinity of the interaction between recombinant S6K1 and CoASy purified from insect cells. CoAsy-His and dephospho CoAsy kinase (negative control) were immobilized to a sensorchip. Various concentrations of recombinant EE-S6K were applied to the chip and the formation of specific protein–protein complexes was monitored, as described in Materials and Methods. The analysis of the sensorgrams clearly demonstrated that S6K specifically interacts with immobilized CoASy (Fig. 3). No specific binding was observed when BSA was applied to the chip (data not shown). Both BSA and S6K displayed negligible binding to the control surface (data not shown). The analysis allowed us to determine the equilibrium dissociation constant (K_D) of the interaction between CoASy and S6K, which was found to be in the range of 180 nM. This study provides further evidence of the specific interaction between S6K and CoA synthase and indicates that it has a relatively high affinity in vitro.

3.4. S6K and CoASy are co-fractionates in the same high molecular weight complexes

Cellular extracts from rat brain were separated by gel filtration chromatography using Superdex 6 column. The individual fractions were analyzed by Western blotting for the presence of CoASy and S6K1. As shown in Fig. 4, CoASy is eluted in two separate peaks: (a) the lower molecular range peak of 60–150 kDa, representing possibly uncomplexed CoAsy, and (b) a high molecular mass peak in the range of 450 kDa, where CoAsy may exist in complex with other cellular proteins. Interestingly, S6K1 is mainly eluted in one high molecular mass peak, whose profile overlaps with the second peak of CoAsy (Fig. 4). These data suggest that CoAsy and S6K1 might be present in the same multienzyme complexes, possibly formed on the outer mitochondrial membrane.

3.5. CoAsy is not a substrate for S6K1 and its activity is not affected by inhibitors of the PI3-K and mTor pathways

The bioinformatic analyses of CoASy protein sequence revealed that it does not possess sequence, corresponding to the S6K substrate motive (RxRxxS/T). However, this motif

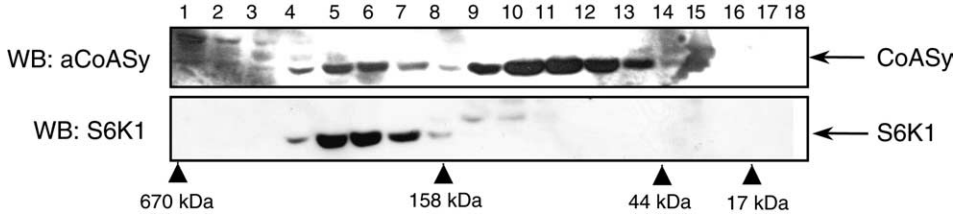

Fig. 4. Gel filtration analysis of CoAsy and S6K1 elution profiles. CoAsy co-fractionates with S6K1 in a high molecular mass complexes. Five hundred microgram of rat brain soluble fractions were separated by gel filtration chromatography on a Superose 6 column. The eluted fractions was separated by SDS–PAGE and analyzed by immunoblotting with the anti-S6K1 and anti-CoAsy antibodies. Apparent molecular weight (mass) of eluted proteins was calculated using gel filtration standard proteins (thyroglobulin, 670 kDa; bovine gamma-globulin, 158 kDa; chicken ovalbumin, 44 kDa; equine myoglobin, 17 kDa; and vitamin B_{12}, 1.35 kDa). Void volume corresponded to the elution of Blue Dextran 2000.

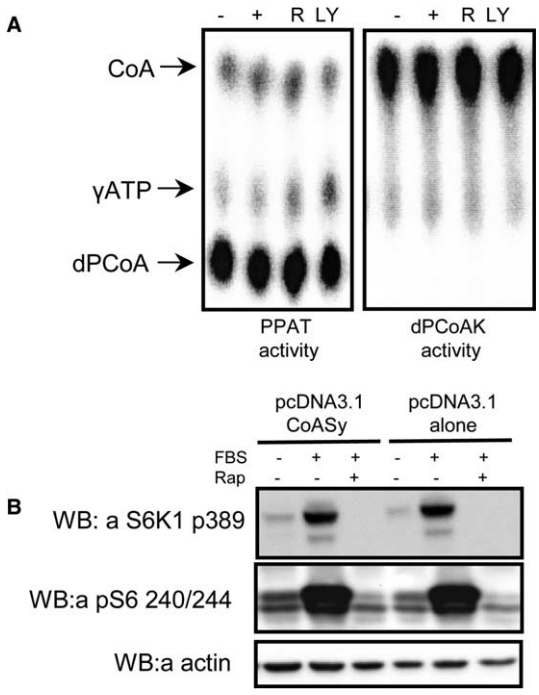

Fig. 5. PPAT and dPCoAK activities are not affected when cells are treated with Rapamycin or LY294002. Overexpression of CoAsy does not affect S6K1 activity or phosphorylation of S6 protein. (A) HEK293 cells were transfected with CoASy-Myc, serum starved and stimulated with FBS in the presence or absence of rapamycin or LY294002. Overexpressed CoAsy was immunoprecipitated with anti-Myc antibody and subjected to in vitro assays measuring PPAT or dPCoAK activities. (B) HEK293 cells were transfected with pCDNA3.1/CoAsy-Myc or vector alone. After transfection, cells were serum-starved for 24 h and stimulated for 60 min with 10% FCS. The supernatants (20 μg of total protein) were analyzed by Western blotting with anti-phospho S6 (pSer 240/244) and anti-phospho S6K (pThr 389) antibodies.

is not so stringent for S6K as in the case of ribosomal protein S6. Therefore, we carried out an in vitro S6 kinase assay using recombinant CoASy and ribosomal protein S6 as substrates. We found no observable phosphorylation of CoA synthase, while S6 protein was readily phosphorylated (data not shown). In addition, the effect of rapamycin and LY294002 on CoAsy was investigated in Hek293 cells transiently transfected with CoAsy-Myc. No significant changes in CoAsy enzymatic activities (4′-phosphopantetheine adenylyltransferase and dephospho-CoA kinase) were found in cells treated with rapamycin and LY294002, when compared with not treated cells (Fig. 5A). In addition, we detected no considerable changes in S6K1 activity when CoASy was overexpressed in HEK293 cells (Fig. 5B).

4. Discussion

Genetic studies in flies and mice strongly indicate that S6K is involved in the regulation of cell growth. The induction of cellular biosynthetic pathways in response to mitogenic stimuli and nutrients is a prerequisite for the accumulation of cellular mass. S6K has been implicated in the augmentation of ribosomal biogenesis and the initiation of protein synthesis. These events are at least partly regulated via the phosphorylation of ribosomal protein S6 or yet unidentified substrate/s of S6K. This report uncovers a novel link between mTor/S6K pathway and another biosynthetic event in the cell, involving CoA synthase. The biochemistry of CoA biosynthesis was uncovered several decades ago, but molecular cloning of its key enzymes including CoA synthase has been only accomplished in the last few years. It is well known that CoA and its derivatives, Acetyl-CoA and Acyl-CoA, are key players in numerous biosynthetic pathways, especially energy metabolism. Therefore, their levels and the ratio in cellular compartments are tightly controlled by known metabolic regulators, such as insulin, glucose, fatty acids and pyruvate, glucagon and glucocorticoids. Disregulation in CoA homeostasis has been observed in several pathological conditions, such as diabetes, Reye syndrome and cancer. So far, very little is known about the regulation of CoA synthase, which controls a rate-limiting step of CoA biosynthesis.

In this study, we present evidence for the specific interaction between CoA synthase and S6K1, employing co-immunoprecipitation of exogenously expressed and native proteins, BIAcore analysis and co-fractionation by gel filtration. It is important to note that only relatively small amount (less than 5%) of transiently overexpressed or endogenous S6K and CoAsy is present in the same complexes. Analysis of deletion mutants indicated that the S6K1/CoAsy interaction is mediated by their C-terminal regions. Interestingly, the ΔN CoAsy mutant binds S6K1 much more efficiently than the full-length protein, indicating a possible interaction between the N- and C-terminal domains or the existence of conformational constrains which could be released by signaling events. We have reported earlier that CoAsy is localized on the outer mitochondrial membrane [15], while S6K1 is mainly present in the cytoplasm and nucleus [4]. Detailed analysis of subcellular fractions and electron microscopy indicated that a small fraction of S6K1 and mTor is also present on mitochondria [10,20]. Taking this into account, we can speculate that membrane-associated CoAsy may form a binding platform for the formation of a multi-enzyme complex, which may include S6K, mTor and other signaling molecules.

We have recently found that both enzymatic activities of CoA synthase are strongly activated by phospholipids [15] and that the lipid-binding domain is localized in the C-terminal region [A. Zhyvoloup, unpublished observation]. Therefore, the interaction between S6K and CoAsy involving their C-terminal domains may modulate lipid-induced activation of CoA synthase. As expected from the bioinformatic analysis, CoAsy was not found to be a substrate for S6K in vitro. So far, we have not been also successful in finding the physiological relevance of S6K/CoA synthase interaction. The activity of CoA synthase was not meaningfully affected by the addition of recombinant S6K in vitro or by treating cells with rapamycin and LY294002. However, we used in this study polyclonal antibodies which have an inhibitory effect on CoAsy enzymatic activities. We are currently making monoclonal antibodies towards CoAsy and are in the process of developing an NMR-based assay for measuring the level of CoA and its derivatives in total cell lysates or cellular organelles. We hope that new antibodies and a CoA detection assay might allow us to elucidate the physiological significance of an established link between mTor/S6K pathway and CoA biosynthetic machinery.

Acknowledgement: The work on this project was supported by grant from the National Academy of Sciences of Ukraine. I. Nemazanyy, G. Panasyuk and A. Zhyvoloup were supported by EMBO Short Term fellowship, FEBS Collaborative Experimental Scholarship for Central & Eastern Europe and the Wellcome Trust fellowship, respectively. We thank Tim Fenton, Richard Foxon and Heike Rebholz for useful discussions and proofreading of the manuscript.

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